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The Chemistry of Allergens. XIII. Ion-Exchange Fractionation of the Cottonseed Allergen and Immunological Properties of the Products

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The Chemistry of Allergens. XIII. Ion-Exchange Fractionation of the Cottonseed Allergen and Immunological Properties of the Products[1]

Introduction

The principal allergen of cottonseed, as contained in CS-1A and subfractions of CS-1A, has been characterized as a complex mixture of relatively low-molecular-weight proteins and polysaccharidic proteins which were classified as natural proteose (1–4). Extensive fractionations of CS-1A resulted in separation of many fractions, the extremes of which were the carbohydrate-free allergen, CS-60C (5), and the carbohydrate-rich allergen, CS-56R-ExD (6). Fraction CS-60C contained 20.4% nitrogen and 0.0% carbohydrate, and CS-56R-ExD contained 4% nitrogen and 87% combined carbohydrate.

Immunochemical studies showed that CS-60C and CS-56R-ExD were of equal potency in eliciting Schultz-Dale responses on a nitrogen basis, and both fractions had the same antigenic specificity. Moreover, in a series of fractions containing widely different proportions of carbohydrate, it was found that the gross anaphylactic shocking capacities were the same when the results were calculated on a nitrogen basis. But the anaphylactic sensitizing capacities were increased significantly by the combined carbohydrate. For example, SD_{50N}[2] for CS-51R (0.9% carbohydrate) was 92 ± 16 μg. In contrast, SD_{50N} for CS-56R-ExD (87.4% carbohydrate) was 7.7 ± 3.6 μg., a 12-fold difference. The threshold quantity of CS-60C nitrogen required to produce positive passive transfer reactions was 0.2 mμg. (5).

Evaluation of available evidence indicated that chemically different components of CS-1A contained within their molecules a portion which

[1] Presented, in part, before the Division of Biological Chemistry at the 131st Meeting of the American Chemical Society, Miami, Florida, April 12, 1957. The previous paper appeared in the *Journal of Allergy* (9).

[2] Median anaphylactic sensitizing dose calculated on a nitrogen basis.

imparted a specificity common to each. It was hoped that this evaluation could be further clarified by isolation of at least two fractions of CS-1A which were separated completely from each other.

Use by other investigators (7) of the cation-exchange resin IRC-50(XE-64) to fractionate basic proteins indicated that the cottonseed allergen, CS-1A, might also be fractionated with this resin. The object of this paper was threefold: namely, to describe the ion-exchange fractionation of CS-1A by chromatography with IRC-50; to demonstrate chemically the complete separation of two active fractions by an ion-exchange method of characterization; and to describe an immunological study of the products obtained.

Experimental

Material Used

CS-1A. Cottonseed allergen, CS-1A, was isolated from depigmented, defatted cottonseed as previously described (1, 2, 8).

CS-13. Fraction CS-13 was prepared from CS-1A by precipitation with picric acid and recovery of active fraction by removal of the picric acid (9). Seventy-eight grams of CS-13, which contained 17.0% nitrogen on an air-dried basis, was obtained from 235 g. of CS-1A.

CS-13E and CS-13D. Details of a typical dialysis of CS-13 have been described (9). Eight dialyzate fractions obtained over a period of 55 days were combined, and fraction CS-13D was isolated from the concentrated combined dialyzate solutions by ethanol precipitation. The fraction remaining inside the membrane was isolated by ethanol precipitation and designated CS-13E. An amount of 41.6 g. CS-13E and 20.4 g. CS-13D was obtained from 76 g. CS-13. CS-13E contained 18.6% nitrogen and 6.1% polysaccharidic carbohydrate, and CS-13D contained 19.4% nitrogen and 0.6% polysaccharidic carbohydrate (ash–water-free basis).

Resin. Amberlite, IRC-50(XE64)[3] was conditioned by the method of Hirs, Moore and Stein (10), except that ethanol was used for washing instead of acetone. The pH of three portions of resin was adjusted to 6.0, 6.5, and 7.0 and equilibrated with 0.1 *N* sodium acetate buffered at corresponding pH values.

Ion-Exchange Fractionation of CS-13E and CS-13D

Fractionation Procedure. The column used was a glass tube 45 mm. in diameter and 190 mm. high with a fritted glass filter sealed to the bottom. The height of the resin in the column was 60 mm. Five grams of allergen fraction, dissolved in 25 ml. of 0.1 *N* sodium acetate buffered at pH 6.0, was introduced into the column. Elution was done with 0.1 *N* sodium acetate solution, pH 6.0, until only traces of allergen came through the column. Analysis of effluent solutions was by an ultraviolet spectrophotometric method described below. Allergen was isolated from effluent fractions by evaporation to a suitable volume and precipitation by 4 vol. ethanol at 5°C. at pH 6.3. The material remaining on the column was eluted with 0.2 *M* ammonium hydroxide until the pH of the last effluent fraction was 10.6. The combined solutions, concentrated at once by air current at room temperature, were precipitated with

[3] The use of a trade name is for identification only and implies no indorsement of the product and its manufacturer.

TABLE I

Ion-Exchange Fractions of CS-13E

Fraction designation	Volume of effluent[a]	Yield[b]	Nitrogen[c]	Carbohydrate[c]
	ml.	*mg.*	%	%
Fractions obtained from 10 g. CS-13E at pH 6.0				
(CS-13E)1	50–100	937	13.1	24.9
(CS-13E)2	100–175	286	16.3	11.3
(CS-13E)3	175–425[d]	253	15.2	29.8
(CS-13E)4	2275–2525	260	17.7	7.3
(CS-13E)5	3025–3625	5200	20.1	0.8
Fractions obtained from 4 g. of (CS-13E)5 at pH 6.5				
(CS-13E)5A	50–150	561	18.7	2.8
(CS-13E)5B	150–225	265	18.9	0.8
(CS-13E)5C	225–450	289	19.5	0.7
(CS-13E)5D	450–950	296	18.9	0.6
(CS-13E)5E	950–2100[e]	370	19.3	0.5
(CS-13E)5F	2900–3400	1548	20.0	0.4
Fractions obtained from 1.3 g. of (CS-13E)5F at pH 7.0				
(CS-13E)5F1	45–135	148	19.1	—
(CS-13E)5F2	135–215	165	20.3	—
(CS-13E)5F3	215–295	93	20.0	—
(CS-13E)5F4	295–455	118	19.8	—
(CS-13E)5F5	455–550[f]	103	19.8	—
(CS-13E)5F6	1100–1350	246	20.1	—

[a] Accumulative volume of effluent. Effluent volumes not recorded were discarded because of negligible allergen content.

[b] Air-dried basis.

[c] Ash- and water-free basis.

[d] Started elution with 0.2 *M* ammonium hydroxide at 2215 ml.

[e] Started elution with 0.2 *M* ammonium hydroxide at 2050 ml.

[f] Started elution with 0.2 *M* ammonium hydroxide at 1050 ml.

ethanol. Allergen isolated from the first 0.2 *M* ammonia eluate was used as starting material for a similar fractionation at pH 6.5, and likewise the allergen obtained from the 0.2 *M* ammonia eluate of the pH 6.5 fractionation was used for the pH 7.0 fractionation.

Ten grams of both CS-13E and CS-13D was fractionated in two 5-g. lots. Results are shown in Tables I and II.

Refractionation of (CS-13E)1. Nine hundred milligrams of (CS-13E)1 was dissolved in 10 ml. of 0.1 *N* sodium acetate buffered at pH 6.0. This solution was introduced into a 22 × 660 mm. column of IRC-50 resin equilibrated with 0.1 *N* sodium acetate buffered at pH 6.0. Elution was done with 0.1 *N* sodium acetate at pH 6.0. Results are shown in Table III.

Refractionation of Combined (CS-13D)1 and (CS-13D)2. Five hundred and fifty milligrams of (CS-13D)1 plus 300 mg. (CS-13D)2 was dissolved in 10 ml. of 0.1 *N* sodium acetate buffered at pH 6.0 and chromatographed similarly to (CS-13E)1. Results are shown in Table III.

TABLE II

Ion-Exchange Fractions of CS-13D

Fraction designation	Volume of effluent[a]	Yield[b]	Nitrogen[c]	Carbohydrate[c]
	ml.	*mg.*	%	%
Fractions obtained from 10 g. of CS-13D at pH 6.0				
(CS-13D)1	50–100	610	17.4	1.2
(CS-13D)2	100–175	350	18.8	0.5
(CS-13D)3	175–450	407	19.0	0.4
(CS-13D)4	450–1050[d]	445	18.7	0.5
(CS-13D)5	3200–3700	4500	20.0	0.15
Fractions obtained from 4 g. of (CS-13D)5 at pH 6.5				
(CS-13D)5A	50–150	811	19.9	0.38
(CS-13D)5B	150–225	342	19.2	0.23
(CS-13D)5C	225–500	474	19.3	0.24
(CS-13D)5D	500–875[e]	233	19.2	0.26
(CS-13D)5E	2975–3325	1100	19.5	0.15
Fractions obtained from 1 g. of (CS-13D)5E at pH 7.0				
(CS-13D)5E1	75–165	104	20.2	0.35
(CS-13D)5E2	165–255	137	19.8	0.23
(CS-13D)5E3	255–320[f]	111	19.9	—
(CS-13D)5E4	1150–1300	215	19.7	0.17

[a] Accumulative volume of effluent. Effluent volumes not recorded were discarded because of negligible allergen content.

[b] Air-dried basis.

[c] Ash- and water-free basis.

[d] Started elution with 0.2 *M* ammonium hydroxide at 2250 ml.

[e] Started elution with 0.2 *M* ammonium hydroxide at 2075 ml.

[f] Started elution with 0.2 *M* ammonium hydroxide at 500 ml.

Spectrophotometric Analysis of Allergen Solutions

The absorbancy index (11) of CS-13 was 67 at 277 mμ as compared to 860 at 220 mμ. Spectrophotometric analysis at 260–280 mμ is not feasible for proteins low in aromatic amino acids like the cottonseed allergen, therefore, 220mμ was used. The interfering effect of general absorption was minimized by elution with sodium acetate solution (U = unbuffered sodium acetate) of the same acetate-ion concentration as the sodium acetate buffer solution used to equilibrate the starting resin. The absorbancy of a solution of 0.3 *N* sodium acetate buffered at pH 5.50 measured against a control containing 0.3 *N* sodium acetate (U) was only 0.035. The blank was negligible as shown in Fig. 2.

Method. Nine milliliters of water in a 12-ml. glass-stopped centrifuge tube was mixed with 1.0 ml. solution containing the allergen in 0.1 *N* sodium acetate (U) or effluent. The blank solution contained 9.0 ml. water and 1.0 ml. of 0.1 *N* sodium acetate (U). Absorption was measured at 220 mμ using a Beckman quartz spectrophotometer, model DU. In analysis by the method of characterization described below, 0.3 *N* sodium acetate (U) solutions were used instead of 0.1 *N*.

Standard Curve. A standard curve was prepared for each fraction using solutions

TABLE III

Ion-Exchange Fractions of (CS-13E)1 and of Combined (CS-13D)1 and (CS-13D)2

Fraction designation	Volume of effluent[a]	Yield[b]	Nitrogen[c]	Carbohydrate[c]
	ml.	*mg.*	%	%
Fractions obtained from 0.9 g. of (CS-13E)1 at pH 6.0				
(CS-13E)1A	120–150	136	10.4	26.2
(CS-13E)1B	150–180	210	13.1	18.5
(CS-13E)1C	180–210	131	15.0	10.0
(CS-13E)1D	210–235	60	16.3	5.4
Fractions obtained from 0.85 g. (CS-13D)1 + (CS-13D)2 at pH 6.0				
(CS-13D)1A	125–185	157	17.5	3.1
(CS-13D)1B	185–245	111	18.0	0.65
(CS-13D)1C	245–320	86	18.3	1.2
(CS-13D)1D	320–400	89	18.4	0.63

[a] Accumulative volume of effluent. Effluent volumes not recorded were discarded because of negligible allergen content.

[b] Air-dried basis.

[c] Ash- and water-free basis.

containing 100, 200, 400, 600, 800, and 1000 μg. allergen/ml. The standard curves of all fractions obeyed Beer's law over the entire range up to an absorbancy of 1.0.

Ion-Exchange Method of Characterization of Allergen Fractions

Resin. Amberlite resin IRC-50(XE-64) was adjusted to pH 5.50 with sodium hydroxide and acetic acid and equilibrated with 0.3 *N* sodium acetate buffered at pH 5.50.

The Column and Eluant Addition Apparatus. The chromatographic apparatus is shown in Fig. 1. The resin column, *A*, was 4 mm. i.d. and 140 mm. long with a coarse Pyrex filter, 10 mm. in diameter, sealed to the lower end. Addition of eluant and control of pressure on the column was accomplished by means of tube *D* with a coarse Pyrex filter and a 10 × 45 mm. tube sealed to the lower end. This Pyrex filter prevented disturbance of the resin surface by the eluant feeding from the reservoir. Tube *C* was connected to tube *B* with a rubber stopper which did not come in contact with solution. Contact of solutions with rubber was avoided because such contact extracted from the rubber a detectable amount of ultraviolet-absorbing material which interfered with analysis. Solutions were saturated with chloroform to prevent bacterial growth.

Filling the Column. Tube *B* was two-thirds filled with 0.3 *N* sodium acetate buffer, pH 5.50. Approximately 1 ml. of a thin slurry of resin in 0.3 *N* sodium acetate, pH 5.50, was added with a dropper, and the resin was allowed to settle. This process was repeated until the height of the resin in column *A* was 140 mm.

Operation of the Column. The excess sodium acetate buffer was removed from tube *B* to the level of the resin in column *A*. The sample solution (up to 50 mg. allergen was dissolved in 2.0 ml. of 0.3 *N* sodium acetate (U), pH 7.8) was added using a funnel with a capillary outlet bent at right angles. Collection and measurement of effluent was started when the sample began to flow into the resin. The sample container was rinsed with 2.0 ml. of 0.3 *N* sodium acetate (U), and this solution was added to the column just as the original sample disappeared into the resin. The washing was re-

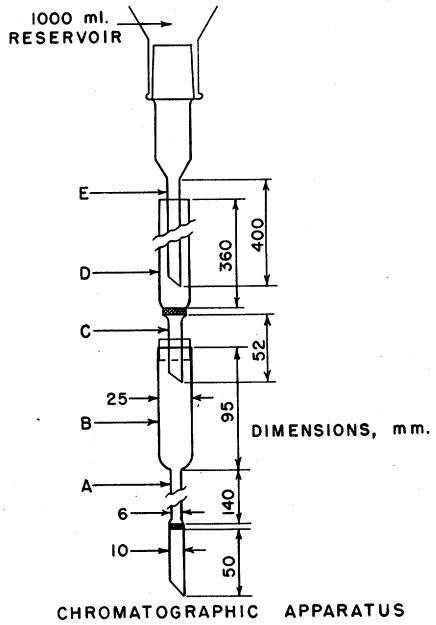

FIG. 1. Chromatographic apparatus used for characterizing cottonseed allergenic fractions. Sample solution is introduced into column *A* which contains Amberlite IRC-50. Eluant solution is fed at constant pressure from the reservoir, through filter tube *D*, into chamber *B* and through column *A*.

peated once. Tube *B* was filled with enough 0.3 *N* sodium acetate (U) to just immerse the lower end of *C*. The eluant addition tube and reservoir were connected, and elution and fraction collection then proceeded automatically. Measurement of effluent was by weight. The rate of elution was from 3 to 6 g./hr. The weight of effluent per sample ranged from 2 to 6 g. The pH of effluent changed from 5.50 to 6.0 at 75 g., to 6.3 at 150 g., and to 6.5 at 200 g.

Recovery of sample was 96% (corrected for blank) in a test in which 50 mg. of CS-13 was chromatographed and 41 samples of effluent were collected. The blank correction was 5%.

DISCUSSION AND RESULTS

Characterizing curves of allergen fractions were obtained by plotting concentration in micrograms/ml. against total weight of effluent. The curves for CS-13, CS-13D, and CS-13E (Fig. 2), indicated that these fractions were complex mixtures.

The resolving power of the column was shown by the curve that was obtained when 25 mg. each of (CS-13E)3 and (CS-13E)5F5 were mixed and the resulting solution was chromatographed (Fig. 3). Essentially complete resolution was obtained by one passage through the column because the peaks of (CS-13E)3 separately and from the mixture were at

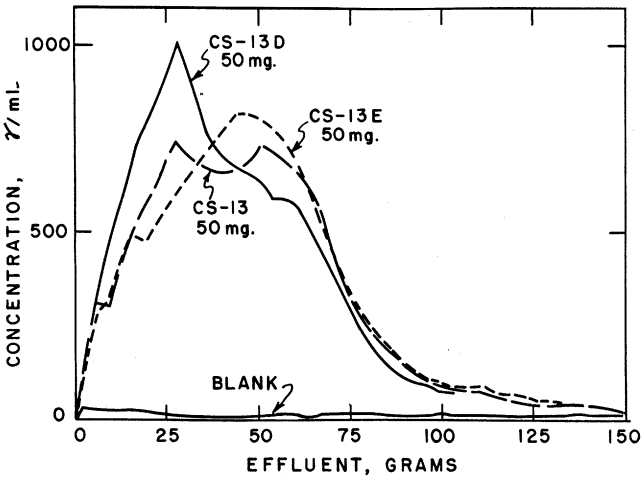

FIG. 2. Characterizing curves of original cottonseed allergen fractions, CS-13, CS-13E, and CS-13D. Fifty-milligram samples dissolved in 2.0 ml. of 0.3 *N* sodium acetate were introduced into the column of IRC-50, equilibrated at pH 5.50, and eluted with 0.3 *N* sodium acetate.

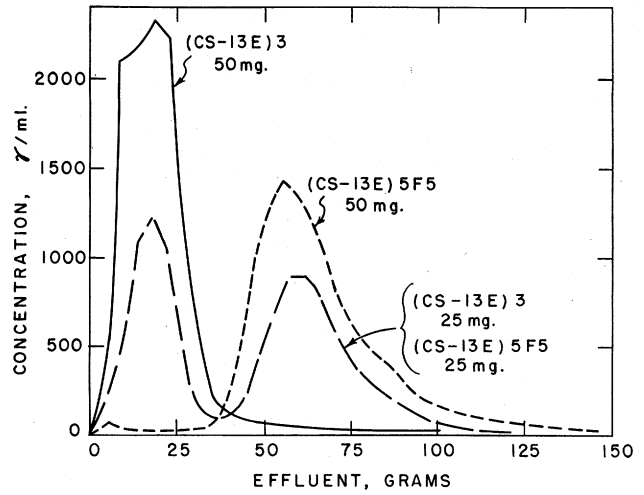

FIG. 3. Resolving power of the column shown by characterizing curves of purified fractions (CS-13E)3 and (CS-13E)5F5, determined singly and with their mixture. Separate 50-mg. samples dissolved in 2.0 ml. of 0.3 *N* sodium acetate were introduced into the column of IRC-50, equilibrated at pH 5.50, and eluted with 0.3 *N* sodium acetate. A single sample consisting of 25 mg. of each fraction was chromatographed likewise.

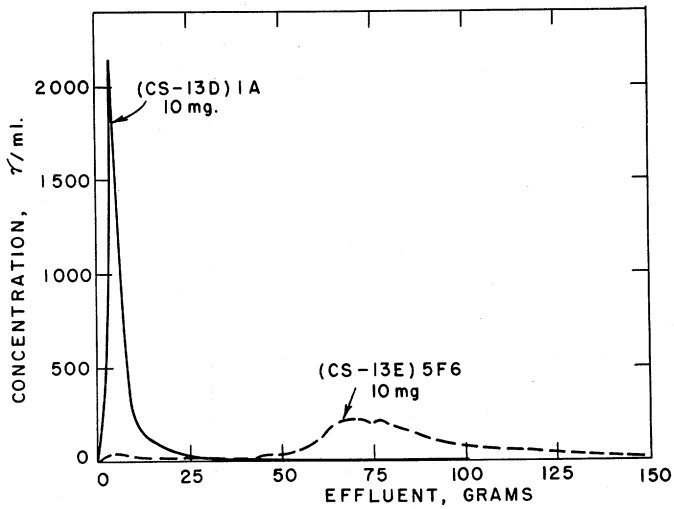

FIG. 4. Characterizing curves of cottonseed allergen fractions, (CS-13D)1A and (CS-13E)5F6 which show maximum separation obtained by dialysis and ion-exchange fractionation. Ten-milligram samples dissolved in 2.0 ml. of 0.3 *N* sodium acetate were introduced into the column of IRC-50, equilibrated at pH 5.50, and eluted with 0.3 *N* sodium acetate.

27–28 g., and the peaks of (CS-13E)5F5 separately and from the mixture were from 56 to 61 g.

The characterizing curves for two fractions (CS-13D)1A and (CS-13E)5F6, which represented the extremes of separation obtained by both dialysis and ion-exchange fractionation, are shown in Fig. 4. It is apparent that (CS-13D)1A and (CS-13E)5F6 were completely free of each other. Fraction (CS-13E)1A gave a characterizing curve similar to that of (CS-13D)1A.

The precipitin capacities of the fractions were determined by the Ouchterlony agar-plate method using CS-13E-rabbit antiserum (12–14). The threshold concentration required to produce precipitin lines was determined using twofold serial dilutions ranging from 200 to 0.08 μg. of fraction nitrogen/ml. for the D series and from 50 to 0.08 μg. of fraction nitrogen/ml. for the E series. The lowest concentration of fraction which gave a visible precipitate was taken as the end point. Results for some key E and D series fractions are shown in Table IV. Of all the fractions obtained, only (CS-13D)1A, (CS-13D)1B, (CS-13D)1C, and (CS-13D)1D gave no precipitin.

The guinea pig sensitizing capacities (SD_{50N}) of CS-13E and two of its subfractions representing maximum separation, (CS-13E)1A and (CS-

TABLE IV

Results of Ouchterlony Precipitin Determination

Fraction	Lowest concentration showing indicated number of lines of precipitate, μg. fraction N/ml., 3 day readings		
	3 lines	2 lines	1 line
CS-13E	50	6	0.35
(CS-13E)1	—	6	6
(CS-13E)5	50	6	0.35
(CS-13E)5A	50	3	0.70
(CS-13E)5F	—	3	0.35
(CS-13E)5F1	—	13	0.70
(CS-13E)5F6	—	—	0.17
(CS-13E)1A	25	13	0.7
(CS-13E)1B	25	6	6
CS-13D	50	6	0.35
(CS-13D)1	—	—	50
(CS-13D)5	50	6	0.35
(CS-13D)5A	—	25	1.5
(CS-13D)5E	50	13	1.5
(CS-13D)5E1	—	50	0.17
(CS-13D)5E4	—	50	0.7
(CS-13D)1A	—	—	[a]
(CS-13D)1B	—	—	[a]

[a] No precipitate from 0.08 to 200 μg. of fraction nitrogen/ml.

13E)5F6, were 92 ± 22, 47 ± 11, and 260 ± 65 μg. of fraction nitrogen, respectively. The corresponding Ouchterlony end points were 0.35, 0.70, and 0.17 μg. of fraction nitrogen/ml., respectively. The SD_{50N} values for (CS-13D)1A and (CS-13D)5E4, two subfractions of CS-13D representing maximum separation, were 850 ± 410 and 550 ± 85 μg. of fraction nitrogen, respectively. By the Ouchterlony method, (CS-13D)1A gave no precipitate up to 200 μg. of fraction nitrogen/ml., and the end point for (CS-13D)5E4 was 0.35 μg. of fraction nitrogen/ml. According to the Ouchterlony tests, (CS-13D)1A contains less than 0.09% (CS-13E)5F6.

(CS-13E)1A, which contained 26% carbohydrate, was 18 times more effective in sensitizing guinea pigs than its counterpart of the D series, (CS-13D)1A, which contained 3% carbohydrate and which gave no precipitin reaction. The greater sensitizing capacity of (CS-13E)1A, as compared to (CS-13D)1A, was attributed to a higher molecular weight due to the combined carbohydrate (6). It is of interest to note that the sensitizing capacity of (CS-13E)5F6 was twice as great as that of its counterpart of the D series, (CS-13D)5E4. This is attributed to the higher molecular weight of the protein component of (CS-13E)5F6 per se because the carbohydrate contents of both of these fractions were negligible.

TABLE V

Sedimentation Constants[a] and Estimated Molecular Weights of Cottonseed Allergenic Fractions

Fraction	$S_{20,w} \times 10^{13}$	Molecular weight[b]
(CS-13D)1A	0.99	5000–12,000
(CS-13D)5A	1.36	—
(CS-13D)5E1	1.49	—
(CS-13D)5E4	1.56	—
(CS-13E)1A	1.04	5000–12,000
(CS-13E)5A	1.59	—
(CS-13E)5F1	1.57	—
(CS-13E)5F6	1.60	10,000–18,000[c]

[a] Sedimentation constants were determined in a Spinco model E analytical ultracentrifuge at 59,780 r.p.m. at room temperature. The determinations were done in pH 5.2 acetate buffer ($\Gamma/2$, 0.1). The concentrations were about 1%.

[b] Diffusion constants were estimated from the sedimentation data.

[c] Estimated $\bar{V}$, 0.75.

The sedimentation constants of several fractions are shown in Table V. The ultracentrifugal patterns obtained in all cases had a single peak, suggesting no gross heterogeneity with respect to higher molecular weight components. It is recognized that heterogeneity with respect to a multiplicity of closely related components could and probably does exist in these samples. Fraction (CS-13E)5F6 is closest to homogeneity because it gave only one line of precipitate by the Ouchterlony method.

The molecular weights of (CS-13D)1A and (CS-13E)1A were calculated to be from 5000 to 12,000 and (CS-13E)5F6 from 10,000 to 18,000 by means of diffusion constants estimated from the sedimentation curves and from estimated partial specific volumes. These values confirm our previous opinion regarding the relatively low molecular weights of the cottonseed and other oilseed allergens of the natural proteose classification.

Attempts to demonstrate differences in specificity of (CS-13D)1A and (CS-13E)5F6 by Schultz-Dale technique were not conclusive, and further study was limited by insufficient material. Study of the immunological relationship of (CS-13D)1A and (CS-13E)5F6 with serum from cottonseed sensitive subjects by the passive transfer technique will be reported elsewhere.

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Summary

The principal allergen of cottonseed, CS-1A, has been fractionated and a method of characterization of subfractions has been described, using cation-exchange resin IRC-50. Two active fractions were obtained which were completely separated from each other as shown by chemical and immunological methods.

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